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REMARKS**I. Preliminary Remarks**

The Applicants thank SPE Gary Kunz and Examiner Christopher Nichols for the courtesy of the personal interview on April 28, 2004 kindly granted to the Applicants' attorney David A. Gass and the undersigned agent.

In paragraph 6 of the Action, the Examiner objected to the recitation "A□" at page 45 (Example 8) of the specification. The recitation "A□" is an obvious typographical error which should refer to "Aβ." The term "Aβ" refers to the amyloid beta peptide that is generated by cleavage of APP at the β-secretase and γ-secretase cleavage sites (see page 1, lines 15-28). The Aβ peptide is referred to throughout the specification including within Example 8 at page 45, line 2 and page 46, lines 3-5. Thus, the amendment does not add new matter to the specification.

In paragraph 7 of the Action, the Examiner objected to claim 239 as depending from canceled claim 86. This is a typographical error that is corrected by the foregoing amendment.

In the foregoing amendment, claims 270-277 have been amended to be directed to "mammalian APP." These amendments are supported throughout the specification and by the originally presented claims.

The sequences set out as SEQ ID NOS: 3 and 4 in the substitute sequence listing, filed on March 26, 2001, are not identical to the sequences set out in Figure 3 as originally filed. Therefore, the attached substitute sequence listing is submitted to revise the sequences of SEQ ID NOS: 3 and 4 so that they are identical to the sequences shown in Figure 3 as filed. These amendments do not introduce new matter, but address an internal inconsistency.

II. Double Patenting

In paragraph 8 of the Action, the Examiner rejected claims 232-240 and 270-278 under the judicially created doctrine of obviousness-type double patenting as being

unpatentable over claims 11-19 of U.S. Patent No. 6,440,698 (denoted as '698). The Applicants traverse this rejection.

The Examiner states claims 11-19 of '698 are not identical to pending claims 232-240 and 270-27. U.S. Patent No. 6,440,698 claims host cells transfected with a nucleic acid sequence encoding APP or a fragment thereof containing a β -secretase cleavage site and two-carboxy-terminal lysine residues. The Applicants dispute that claims 11-19 of '698 are all relevant to double patenting. However, a terminal disclaimer is submitted herewith to expedite prosecution rendering the obviousness type double patenting rejection moot.

III. The rejection under 35 U.S.C. § 112, first paragraph for lack of enablement should be withdrawn.

In paragraph 11 of the Action, the Examiner rejected claims 232-240 and 270-278 under 35 U.S.C. § 112, first paragraph, alleging that the specification does not enable one of skill in the art to make or use the invention commensurate in scope with the pending claims. In particular, the Examiner stated that the specification fails to provide any guidance for the isolation and characterization of any isoform of APP that is encompassed by the claims. The Applicants traverse this rejection.

To determine if the claims require undue experimentation, the factors set out in *In re Wands et al.*, 858 F2d. 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Circ. 1988) are considered including: 1) the breadth of the claims, 2) the nature of the invention, 3) state of the prior art, 4) the level of one of ordinary skill, 5) the level of predictability in the art, 6) the amount of direction provided by the inventor, 7) the existence of working examples and 8) quantity of examination needed to make or use the invention based on the content of the disclosure.

The nature of the invention is the addition of carboxy di-lysine to an APP polypeptide or fragment of APP. Carboxy di-lysine is an endoplasmic reticulum retention signal. (Cossen & Letourneau, *Cold Spring Harbor Sympos. Quant. Biol.* 60: 113-117, 1995). The addition of two lysines to the carboxy terminus of APP increases the time APP remains within the endoplasmic reticulum and thereby increases its availability for cleavage by the β -secretase pathway. The specification demonstrates that the addition of the carboxy di-lysines increased the efficiency of cleavage of the modified APP at the β -secretase site. The breath

of the pending claims encompasses the genus of mammalian amyloid protein precursor (APP) polypeptides bearing such a modification, and polynucleotides that encode the modified APP. As discussed in detail below, mammalian APP sequence information for numerous species, including isoforms and mutations, were known in the art at the time of filing. The level of skill in the art was high.

Sequence information for numerous representative species of the genus of molecules known as "mammalian amyloid precursor protein (APP)" was well known in the art at the time of filing. Therefore, following the teachings in the application, one of skill in the art would have been able to make and use the modified APP molecules of the invention. The specification provides the citations for the references disclosing the main human APP isoforms (APP751; Ponte *et al.*, *Nature* 331: 525-527 1988 and Tanzi *et al.*, *Nature* 331:528-530; APP770 Kitiguchi *et al.*, *Nature* 331:530-532, 1988) and certain known mutations (page 17, lines 9-21).

The references cited by the Examiner in the Action (Haas *et al.* *Biochemica et Biophysica Acta* 1343(1):85-94, 1997 and Ghiso *et al.* *Biochem J.* 288(3):1053-1059, 1992) provide evidence that the state of the art surrounding APP was sophisticated at the time of filing. Both, Haas *et al.*, and Ghiso *et al.*, identify useful fragments of APP. Ghiso *et al.* also provides evidence that antibodies to detect APP polypeptides and fragments thereof were well known in the art at the time of filing (see page 1056, column 1). These references provide evidence that the level of skill in the art was high at the time of filing and in combination with the teachings in the specification, one of skill in the art is enabled to modify any known APP isoform with two carboxy lysines.

In addition, Johnstone *et al.* (*Molecular Brain Research* 10:299-305,1991), cited by the Examiner, demonstrates that the known mammalian APP isoforms are highly conserved. Johnstone *et al.* taught the amino acid sequence from 10 mammalian APP polypeptides in 1991. Thus, the genus of mammalian APP was reasonably represented in the art in 1998, the effective filing date of the present application. In particular, Johnstone *et al.* states "the amino acid sequence of the β /A4 peptide in dog and polar bear is identical to the human and monkey sequences... We have also shown that the amino acid sequence of rabbit, cow, sheep, pig and guinea pig is identical to the human sequence." (see Johnston *et al.* page 303, column 2). The peptide results from beta and gamma secretase cleavage of APP, and

one skilled in the art would appreciate the significance of this high level of sequence conservation in the region of APP in the context of a beta secretase invention.

The various isoforms of APP and homologues thereof may not be identical in amino acid sequence but do share a common structure, and this common structure guides one of skill in the art to characterize these molecule as isoforms and homologs of APP. These structural characteristic of APP were known at the time of filing and are as follows: signal sequence followed by an acid rich region, protease inhibitor motif, N-glycosylated region, transmembrane domain and a small cytoplasmic domain (see Soto *et al.*, *J. Neurochem.* 63: 1191-1198, 1994; Appendix A). Thus the existence of various isoforms does not increase the unpredictability of the art. On the other hand the similar structural aspects, the high degree of conservation of the amino acid sequence and the continued sensitivity to aspartyl protease cleavage indicates the level of unpredictability is low. The specification need not disclose what is well known in the art, and preferably what is well-known is omitted. (See *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1132 (Fed. Circ. 1991) or MPEP § 2146.08) Thus, the nature of the prior art and the level of predictability in the art at the time of filing is evidence that the claims were enabled by the specification in light of the knowledge of one of ordinary skill in the art.

The Examiner should consider the amount of direction provided by the inventor in the specification and the presence of working when determining if the genus of mammalian APP is enabled. The specification provides the nucleotide and amino acid sequence of APP695 and provides a working example demonstrating how to modify APP695 by inserting two lysines at the carboxy terminus (Example 6; page 38, line 30 through page 42, line 15). The specification further teaches that the addition of di-lysine residues to the carboxy terminus increases the efficiency of cleavage at the β -secretase cleavage site (page 41, lines 28 through page 42 line 15). The working examples also demonstrate that APP695 containing the London mutation or the Swedish mutation in addition to carboxy di-lysines further increases processing at the β -secretase cleavage site as compared to wild type APP (page 46, line 17 through page 49, line 15). The references listed in the specification teach the sequences of the main human APP isoforms, therefore the teachings in Example 6 and 8 allow one of skill in the art to modify any APP isoform with the addition of carboxy di-lysines. Further, the Applicants contemplated modifying APP isoforms APP751 and APP770 by adding two carboxy lysines (page 29, lines 19-23). Thus, the specification provides one of

skill in the art with the necessary guidance to understand how to make and use the claimed invention.

The Examiner stated that the specification fails to provide any guidance for successful isolation and characterization of any isoform of APP except APP695 and therefore it would require undue experimentation to carry out the claimed invention. (pg. 6, ¶14 of the Action) However, as the references cited in the specification and those references cited by the Examiner in the present office action demonstrate, at the time of filing the methods to isolate and characterize APP molecules were well known in the art. The pending claims are directed to adding carboxy di-lysines to APP molecules. As discussed above, many members of the genus of mammalian APP polypeptides had been characterized in the state of the art at the time of filing. The evidence cited by the Examiner in the Action, such as Johnstone *et al.*, indicates the genus of mammalian APP was well characterized at the time of filing and the known species are highly conserved. The knowledge in the art in combination with the methods taught in the specification that allow one of skill in the art to identify APP isoforms cleaved at the β -secretase cleavage site indicates that the identification of new member of the genus is likely to be successful.

For the foregoing reasons, the genus of mammalian APP polypeptides recited in the claims are enabled by the specification and the state of the art at the time of filing. Thus, the rejection of claims 230-242 and 270-278 under 35 U.S.C. § 112, first paragraph, for lack of enablement should be withdrawn.

IV. The rejection under 35 U.S.C. § 112, first paragraph, for lack of adequate written description should be withdrawn.

In paragraph 22 of the Action, the Examiner rejected claims 232-240 and 270-278 under 35 U.S.C. § 112 first paragraph, for lack of adequate written description in the specification. In particular, the Examiner alleged the claims are drawn to a genus of polypeptides that does not require any particular conserved structure or other distinguishing features. The Applicants traverse this rejection.

The Examiner asserts that the specification does not identify any particular portion of the structure to be conserved nor does it provide a structure/function correlation. (pg. 9, ¶24 of the Action) As discussed in detail above, the genus of APP molecules, although encompassing various isoforms, was well characterized in the art at the time of

filing. The Examiner asserted that the specification adequately described one APP species, APP695 containing the wildtype β -secretase cleavage site and the Swedish mutation. However, at page 17, lines 9-21, the Applicants describe the three main human APP isoforms (APP695, APP751 and APP770) and provide citations to the references which teach the nucleotide and amino acid sequence of each isoform. Attached as Appendix B are the references that are referred to at page 17 of the specification, which describe the cloning and sequencing of the main APP isoforms. As illustrated in Tanzi *et al.* (*Nature* 331:528-530, 1988) and Kitaguchi *et al.*, (*Nature* 331:530-532, 1988 in Figure 1), APP751 and APP770 are identical to APP695 with the exception of a 56 or 75 amino acid insertion immediately after Arg288. Tanzi *et al.* provides an alignment of APP751 with APP695 to illustrate the small difference in amino acid sequence. All of the isoforms share the same common beta secretase cleavage site and transmembrane region. Even though the working examples in the specification were only carried out with APP695, it is expected the di-lysine modification will increase cleavage of the isoforms similar to the effect on APP695 due to the high level of amino acid conservation.

As evidence of the broad genus, the Examiner again cited to Johnstone *et al.* (*Molecular Brain Research* 10:299-305, 1991) which demonstrates the various mammalian homologs of APP are not identical. (pg. 9, ¶24 of the Action) As discussed above, Johnstone *et al.*, demonstrates the high level of conservation between mammalian forms of APP. Johnstone *et al.* taught that sequence information from at least 10 mammalian APP polypeptides was known in 1991 and amino acid sequence of these mammalian APP polypeptides are highly conserved. An alignment of 10 full length mammalian APP amino acid sequences is attached hereto as Appendix C. This alignment demonstrates that these sequences are highly conserved. In addition, of the 10 full length amino acid sequences displayed in Appendix C, five were known prior to the time of filing. Thus, the genus of mammalian APP was reasonably represented in the art in 1998, the effective filing date of the present application. Moreover, the art indicates there is not substantial variation within the species of the genus of mammalian APP polypeptides. Finally, it worth reiterating that the Applicants are not claiming the genus of mammalian APP *per se*, but rather, APP with a particular type of modification. As the specification provides the three main human isoforms and two common mutations, a representative number of species are disclosed in the specification and were characterized in the art at the time of filing. Written description of a

claimed genus is satisfied through a sufficient description of a representative number of species. (See *Regents of Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1569, 43 U.S.P.Q.2D (BNA) 1398 (Fed. Circ. 1997 or MPEP §2163 II. 3 (ii)).

In addition, the state of the art at the time of filing demonstrates that one of skill in the art would understand that the Applicants were in possession of the genus of mammalian APP polypeptide at the time of filing. The structural characteristics of APP were known in the art and one of skill would recognize the necessary structural characteristics of this genus. An APP polypeptide is known to "start with a leader sequence (signal peptide), followed by a cysteine-rich region, an acid-rich domain, a protease inhibitor motif, putative N-glycosylated region, a transmembrane domain and finally a small cytoplasmic region." (see Soto *et al.*, *J. Neurochem.* 63: 1191-1198, 1994, p1191 column 2; Appendix A). Thus one of skill of the art would recognize that the Applicants were in possession of the claimed modified APP molecules.

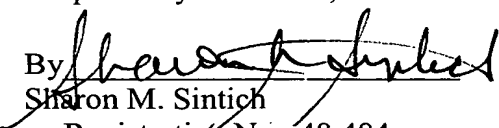
For foregoing reasons, the genus of mammalian APP molecules is supported by an adequate written description in the specification. Thus, the rejection of claims 230-240 and 270-278 under 35 U.S.C. § 112, first paragraph for lack of adequate written description should be withdrawn.

CONCLUSION

In light of the forgoing amendment and remarks, the Applicants believe claims 232-240, 270-278 and 301-303 are in condition for allowance and early notice thereof is earnestly solicited.

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Short Review

Structural Determinants of the Alzheimer's Amyloid β -Peptide

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Abstract: The hallmark event of Alzheimer's disease (AD) is the deposition of amyloid as insoluble fiber masses in extracellular neuritic plaques and around the walls of cerebral blood vessels. The main component of amyloid is a hydrophobic peptide, named amyloid β -peptide (β A4), which results from the processing of a much longer membrane amyloid precursor protein (APP). This review focuses on the structural features of β A4 and the factors that determine β A4 insolubilization. Theoretical and experimental studies of the primary structure of β A4 have shown that it is composed of a completely hydrophobic C-terminal domain, which adopts β -strand structure, and an N-terminal region, whose sequence permits different secondary structures. In fact, this region can exist as an α -helical or β -strand conformation depending on the environmental condition (pH and hydrophobicity surrounding the molecule). The effects of pH and hydrophobicity on β A4 structure may elucidate the mechanisms determining its aggregation and amyloid deposition in AD. **Key Words:** Amyloid— β -structure—Senile plaque—Dementia.

J. Neurochem. **63**, 1191–1198 (1994).

Alzheimer's disease (AD) is the most common form of dementia in adults. It produces a progressive loss of intellectual function and appears both sporadically and in an autosomal dominant (familial) form (Katzman and Saitoh, 1991; Yankner and Mesulam, 1991). Approximately 10% of the population over 65 years old is affected by this form of progressive dementia (Katzman, 1986; Inestrosa, 1992).

The major neuropathological changes in brains of AD patients were observed first by Alois Alzheimer (1907) and include neuronal cell death accompanied by the presence of abnormal intra- and extraneuronal proteinaceous deposits (Katzman, 1986). Intracellularly, bundles of paired helical and straight filaments composed largely of phosphorylated ubiquitin-conjugated τ protein are referred to as neurofibrillary tangles (Kosik, 1991). Extracellularly, amorphous insoluble

aggregates of proteinaceous debris termed "amyloid" appear in the form of senile plaques or neuritic plaques and cerebrovascular amyloid deposits (Glenner, 1980).

Amyloid deposits are a major feature of AD brain. Several lines of evidence suggest that the amyloid may play an important role in early pathogenesis of AD (Hardy and Higgins, 1992; Inestrosa and Soto, 1992). The main component of amyloid is a 4.1–4.3-kDa hydrophobic peptide, named amyloid β -peptide (β A4), that is codified in chromosome 21 as part of a much longer amyloid precursor protein (APP; Muller-Hill and Beyreuther, 1989). The APP starts with a leader sequence (signal peptide), followed by a cysteine-rich region, an acidic-rich domain, a protease inhibitor motif, a putative *N*-glycosylated region, a transmembrane domain, and finally a small cytoplasmic region (Fig. 1A). The β A4 sequence begins close to the membrane on the extracellular side and ends within the membrane. Two-thirds of β A4 faces the extracellular space, and the other third is embedded in the membrane (Kang et al., 1987; Dyrks et al., 1988).

APP matures rapidly through a constitutive secretory pathway as shown in cultured cells (Weidemann et al., 1989). APP is cleaved within β A4 (Sisodia et al., 1990) between residues 16 (lysine) and 17 (leucine) (Esch et al., 1990), as shown by analysis of the fragments secreted into the medium and those retained in the plasma membrane of the cells (Wang et al., 1991). Recent studies indicate that APP is cleaved while sitting in the plasma membrane by a membrane-bound endoprotease (APP secretase) and that the specificity of peptide bond hydrolysis is largely independent of the primary sequence of the precursor. The α -helical conformation and the distance (12–13 amino acid resi-

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The first two authors contributed equally to this review.

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; β A4, amyloid β -peptide.

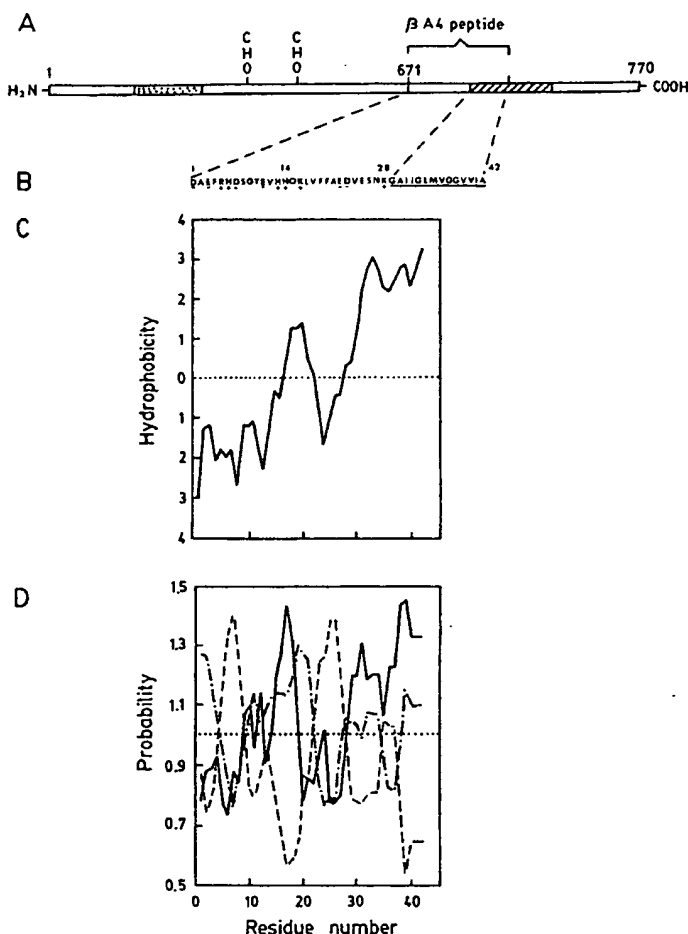


FIG. 1. A: APP molecule scheme showing its transmembrane domain (hatched box), Kunitz domain (dotted box), the β A4 region, and glycosylation sites (CHO). B: The primary structure of β A4 peptide identifying its transmembrane domain (underlined) and the charged amino acids (+/-). C: The hydrophobicity profile of β A4 peptide as obtained using the Kyte and Doolittle (1982) method. D: The secondary structure prediction analysis. The probabilities of α -helix (.....), β -strand (—), and β -turn (---) were calculated using the Chou and Fasman (1978) method.

dues) of the hydrolyzed bond to the plasma membrane have been suggested as the main determinants for proteolysis by APP secretase (Sisodia, 1992).

Current hypotheses regarding AD pathogenesis focus on protease/protease inhibitor imbalances that cause the deposition of β A4 (Abraham et al., 1988; Alvarez et al., 1992; Hardy and Higgins, 1992). Among the potential APP secretases, a cathepsin B (Tagawa et al., 1991), a macropain-like multicatalytic proteinase (Ishiura et al., 1989), and a protease activity associated with acetylcholinesterase (Small et al., 1991a) can cleave APP, albeit at a nonamyloidogenic site. Whether proteolytic activity is a property of acetylcholinesterase or of copurifying substances remains to be determined (Checler, 1990; Small et al., 1991b). Recent studies by Mesulam and coworkers indicate that protease inhibitors (carboxypeptidase inhibitor and bacitracin) inhibit acetylcholinesterase associated with senile plaques (Wright et al., 1993). This latter result opens the possibility that AD-related acetylcholinesterase could participate in the altered protein processing

of APP and eventually in the pathogenesis of the disease.

HOW IS β A4 GENERATED?

β A4 fragments may be produced by an alteration of the specificity of an APP secretase or by an AD-specific membrane disturbance that makes APP accessible to proteinases (Ishiura, 1991). An alternative breakdown yields fragments (8–12 kDa) that constitute the β A4 sequence, and thus have the potential of forming amyloid (Estus et al., 1992). The potentially amyloidogenic derivatives can be produced in normal human cells, including brain cells. The fragments are probably formed in lysosomes, as inhibition of lysosomal enzymes (leupeptin and ammonium chloride) decreases the formation of amyloidogenic fragments (Estus et al., 1992). APP is also present in clathrin-coated vesicles, which are involved in the transport of many proteins via the endosomal/lysosomal pathway (Nordstedt et al., 1993). In addition, the presence of mature APP

and an extensive array of β -amyloid peptides have been shown in purified lysosomes (Haass et al., 1992a). Therefore, the available evidence indicates that APP is normally processed by both the constitutive secretory and the endosomal/lysosomal pathways (Inestrosa and Soto, 1992). On the other hand, recent studies using inhibitors of Golgi processing and immunocytochemical and subcellular fractionation techniques have suggested that the β -amyloid peptide could be produced in the secretory pathway at the Golgi complex (Caporaso et al., 1992; Busciglio et al., 1993; Haass et al., 1993).

Soluble β A4 is released by healthy cells in culture, and also has been recovered from human and animal cerebrospinal fluid (Seubert et al., 1992; Shoji et al., 1992). The soluble peptide is 40 amino acids long, whereas the main component of β -amyloid in plaques is two to three amino acids longer at the C terminus (Prelli et al., 1988). This fact makes it difficult to think that soluble β A4 is the same as plaque β A4, although it may reflect the occurrence of minor and longer components in the soluble β A4, which are selectively deposited. In this context, it has been proposed that the amyloid formation is a nucleation-dependent phenomena (Jarrett et al., 1993). In fact, kinetic studies of the amyloid formation have shown that the length of the C terminus is a critical determinant of the rate of amyloid deposition. Thus, amyloid formation by the kinetically soluble peptides can be nucleated or "seeded" by peptides, including a longer C terminus (Jarrett and Lansbury, 1993; Jarrett et al., 1993).

PHYSICAL PROPERTIES OF THE AMYLOID PROTEINS

The deposition of a fibrous protein aggregate is not a phenomenon unique to AD. In fact, amyloid deposits are characteristic of many diseases, including several forms of systemic amyloidosis (Castaño and Frangione, 1988; Price et al., 1993).

The amyloid deposits of various origins are most insoluble under physiological conditions. They have in common birefringent properties after staining with Congo red, an unbranched fibrillar ultrastructure as determined by electron microscopy, and a distinct x-ray fiber diffraction pattern (Glenner, 1980; Merz et al., 1983; Kirschner et al., 1986). However, none of these experimental approaches provides enough information to establish the molecular structure of the amyloid at a high level of resolution. Furthermore, because amyloid is insoluble but not crystalline, the methods commonly used to determine protein structure, e.g., nuclear magnetic resonance and x-ray crystallography, cannot be used in this case.

No common sequence characterizes all amyloid proteins. Prediction of secondary structures and estimations of the hydrophobicity indicate some similarities among amyloid aggregates of different origins. Many amyloidogenic proteins have a large amount of β -sheet

structure in their soluble form, and they contain sequences rich in valine or isoleucine, amino acids not normally found in helical or random coil secondary structures. Furthermore, *in vivo* studies of protein folding indicate the existence of an almost perfect correlation between insolubilization and formation of β -sheet structures (Haase-Pettingell and King, 1988). However, insoluble peptides with little or no β -sheet structure have been observed (Lansbury, 1992). The insolubility of amyloid deposits may be due to similar intermolecular array rather than to a common secondary structure (Lansbury, 1992). Therefore, the physical bases for protein insolubility are unclear at the present time.

GENERAL STRUCTURAL CHARACTERISTICS OF β A4 PEPTIDE

The first partial amino acid sequence of β A4 was reported by Glenner and Wong (1984). They described the purification of amyloid protein from cerebrovascular deposits and its identification with Congo red dye. The elution of this protein from a Sephadex G-100 column showed that it had a molecular mass of 4,200 Da. Its HPLC purification gave two peaks having identical amino acid composition for the first 24 amino acids; surprisingly, these sequences did not present homology with known proteins. With the advent of successful protocols for solubilizing plaque core amyloid, a peptide was isolated from neuritic plaques whose size and N-terminal sequence were similar to those of the amyloid β A4 on cerebrovascular deposits (Masters et al., 1985).

The complete sequence of β -amyloid peptide was later described by Kang et al. (1987). The peptide size varies depending on its origin. Thus β A4 from senile plaques has a longer C-terminal domain (43 amino acids long) than that recovered from cerebrovascular deposits, which is 40 amino acids long and also more soluble (Prelli et al., 1988). Residues 29–43 of β A4, which correspond to its C terminus, are embedded in the plasma membrane, and the sequence is rich in the hydrophobic amino acids valine and isoleucine (Fig. 1B). According to Chou-Fasman analysis (Chou and Fasman, 1978), leucine and valine are often found in proteins with β -sheet structure and rarely found in helical or random coil secondary structures. Furthermore, the presence of glycine residues can stabilize amyloidogenic structures by means of hydrogen bonds (Lansbury, 1992).

The hydropathy plot, using the method of Kyte and Doolittle (1982), indicates a great hydrophobicity in the last 10 amino acids at the C terminus of β A4, as well as in the sequence between residues 17 and 21 (Fig. 1C). Chou-Fasman analysis suggests the existence of α -helix, β -turn, and β -strand types in the secondary structures (Fig. 1D). The probability of finding β -strand conformation in β A4 is high within the C-terminal region beyond residue 28; the probability is lower

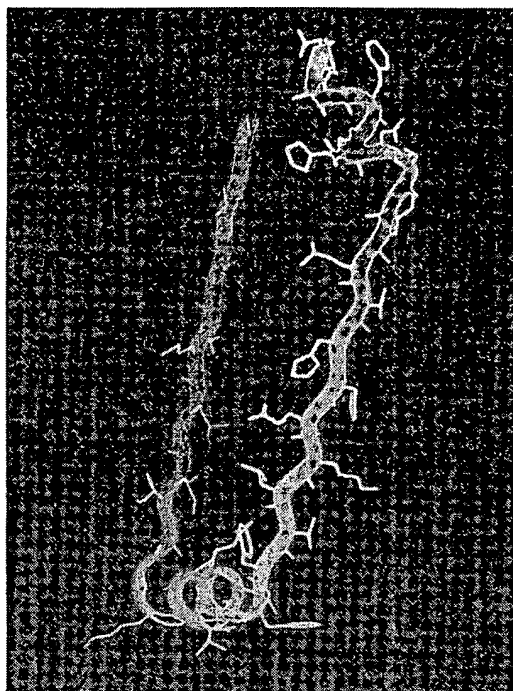


FIG. 2. Model of the $\beta A4$ tridimensional structure obtained by the computer program Insight II of Biosym Technologies. This program places each amino acid in some secondary structure motif (i.e., α -helix, β -strand, β -turn, or random coil) according to the probability calculated by the Chou and Fasman (1978) method. Then the program diminishes the conformational energy until reaching the optimal structure with the minimum energy.

between residues 9 and 21. The latter region is more likely to display α -helix structure. There are also two probable β -turns between residues 6 and 8 and between residues 23 and 27. With all this information, we built a model for the tridimensional structure of $\beta A4$ in its soluble form (Fig. 2) using the computer program Insight II, which predicts the secondary structure by Chou and Fasman's method (1978). It assigns this structure to the peptide sequence and reduces the energy associated with the conformational state until it reaches the structure with the minimum energy. The theoretical structure of $\beta A4$ consists of two β -strands separated by a turn that form a small α -helix. This structure could be the basic unit for fibril formation through hydrogen bonding between the N-terminal β -strand of one peptide and the C-terminal β -strand of the other peptide.

In vivo, the aggregation of $\beta A4$ leads to the formation of insoluble amyloid fibrils having a diameter of approximately 40–90 Å and a length of 76 Å or more. The amyloid fibril from senile plaques or from cerebrovascular deposits appear very similar under the electron microscope (Kirschner et al., 1986). They also stain with Congo red, as do other proteins with β -

pleated sheet structure, i.e., they emit green birefringence under polarized light, even though this property is not specific for proteins with cross β -fibrillar structure (Lansbury, 1992).

By means of x-ray diffraction spectroscopy, it has been possible to confirm the β -pleated sheet structure of $\beta A4$ within the amyloid fibril (Kirschner et al., 1986). Using this technique, the distance between the polypeptide chains was found to be 4.76 Å and the distance between sheets was 10.6 Å. The antiparallel disposition appeared stabilized by hydrogen bonding in the same axis of the fiber.

STRUCTURAL STUDIES WITH SYNTHETIC PEPTIDES

A fruitful approach to studying the structure and solubility of $\beta A4$ has been the synthesis of fragments or the complete sequence of this peptide initiated by Frangione and coworkers (Castaño et al., 1986; Gorevic et al., 1987). Short fragments of $\beta A4$ have proved interesting from a physicochemical point of view, as they aggregate to resemble the naturally occurring amyloid of 4.2 kDa. In fact, sequences 1–28, 12–28, and 14–28 lead to amyloid fibril formation in vitro (Gorevic et al., 1987). Under the electron microscope, their morphology was comparable to fibrils of senile plaques. The staining with Congo red and the x-ray diffraction pattern also yielded similar patterns, homologous to those of proteins that share a β -sheet secondary structure. Studies using synthetic fragments of the $\beta A4$ sequence suggest that the markers of β -pleated conformation (fibrillar ultrastructure, birefringence with Congo red, x-ray diffraction pattern) are present in a minimum stretch of 15 residues (14 to 28). The following hydrophobic domain proved not necessary for fibril formation, but may help make $\beta A4$ more insoluble (Gorevic et al., 1987). In fact, it has been shown recently that the C terminus of $\beta A4$ determines the rate of amyloid fibril formation rather than the stability and structural properties of the amyloid (Jarratt et al., 1993).

Using synthetic peptides, a β -turn was shown between residues 26 and 29 (Hilbich et al., 1991). This was accomplished by the differential stabilization of the β -turns in different positions by means of disulfide bridges.

Another approach has been to alter the primary sequence of the fragments and to study the emerging properties. Thus, the substitution of only lysine 16 for an arginine in the fragment 1–28 gives rise to a morphologically different fibril that retains its β -sheet structure. Nevertheless, the x-ray diffraction pattern suggests that this fibril contains a larger number of β -pleated sheets in the same fibril diameter (Kirschner et al., 1987). To study the influence of the secondary structure of $\beta A4$ on its solubility properties, we analyzed the effect of the substitution of valine 18 (an amino acid-forming β -sheet) for an alanine (an amino

acid-forming α -helix) on the β A4 aggregation. The modified peptide became significantly less aggregated than β A4 unmodified (C. Soto and N. C. Inestrosa, manuscript submitted).

The substitution of glutamic 22 by glutamine, which is the mutation responsible for the generation of the Dutch-type amyloidosis cerebrovascular hemorrhagic disease (Levy et al., 1990), yields a peptide having an increased ability to form fibrils (Wisniewski et al., 1991). This peptide generates fibrillar structures in 1 h compared with the 24 h necessary for wild-type peptide.

On the other hand, the replacement of hydrophobic for hydrophilic residues in the β A4 sequence impairs the formation of fibrils (Hilbich et al., 1991). This suggests that the insolubility of β A4 is mainly due to its hydrophobic residues. The same idea is supported by studies using synthetic peptides of the C-terminal side of β A4, namely, residues 26–33 and 34–42 (Halverson et al., 1990). Interesting differences emerged in their solubility and conformational properties. For example, peptide 26–33 was freely soluble in water, whereas peptide 34–42 was insoluble in aqueous media and also in the presence of denaturing agents. Although both peptides were able to form fibrils, their morphologies were different. Peptide 26–33 assembled into thin, very long flexible fibers having a uniform diameter of 50–55 Å. In contrast, peptide 34–42 formed rigid fibers of various diameters (0.01–3.00 μ m or greater) and appeared as twisted ribbons (Halverson et al., 1990). However, x-ray diffraction analysis revealed that the peptide conformation in the fibrils was β -pleated. A correlation between solubility and β -structure formation was inferred from Fourier transform infrared spectroscopic studies. The peptide 26–33, when dissolved in water, existed as a random coil, whereas the water-insoluble peptide 34–42 possessed antiparallel β -sheet structure in the solid state. Solubilization of peptide 34–42 in organic media resulted in the disappearance of β -structure (Halverson et al., 1990).

Recently, elements of the extracellular matrix have been suggested to play a role in the aggregation of β A4 (Fraser et al., 1992; Brandan and Inestrosa, 1993). The observation of fibril formation with various peptides (11–28, 13–28, 15–28, and 11–25) of β A4 and the determination of their x-ray diffraction pattern revealed the influence of sulfate ions in the extensive lateral aggregation and axial growth of the synthetic peptides into "macrofibers" (Fraser et al., 1992). This effect, seen at sulfate concentrations between 5 and 50 mM, was specific because it could not be duplicated with other ions. This suggests that sulfate itself and not its polyanionic character is required to promote aggregation. Highly sulfated molecules within the extracellular matrix, such as proteoglycans, may provide a high-affinity surface for the direct deposition of existing amyloid fibrils at the extracellular matrix of brain tissue (Brandan and Inestrosa, 1993). The best candi-

dates to participate in electrostatic interactions between peptide 11–28 and sulfate may be any of the four basic residues within the peptide sequence: His 13, His 14, Lys 16, and Lys 28. This interaction and the macrofiber formation were inhibited by pH values higher than 7.

RELATIONSHIP BETWEEN β A4 STRUCTURE AND THE EXTRACELLULAR pH

The endosomal/lysosomal pathway for processing of APP (Golde et al., 1992; Haass et al., 1992a; Nordstedt et al., 1993) does not exclude β A4 generation as a physiological event. Thus, β A4 may be produced as a soluble peptide which, by changes in its environment, may precipitate. Barrow and Zagorski (1991) have reported that local variations in pH or temperature, or the presence of elements such as proteoglycans, could induce the aggregation of β A4. Soluble β A4 is released by human mononuclear leukemic cells (Shoji et al., 1992). This peptide is 40 amino acids long (Haass et al., 1992b), i.e., shorter than the peptide deposited in the neuritic plaques by two to three amino acids (Prelli et al., 1988).

Recent studies elucidate the mechanisms that cause soluble β A4 to form amyloid fibrils. Peptides derived from β A4 or the complete sequence itself show different conformation and solubilities depending on the pH of the solution (Barrow and Zagorski, 1991; Barrow et al., 1992; Burdick et al., 1992; Zagorski and Barrow, 1992). Specifically, fragment 1–28 was found as a monomeric α -helical structure when solubilized in a membrane-mimicking solvent that encourages intramolecular hydrogen bonding. This soluble peptide unfolds to a partly random coil structure with increasing temperature at pH 1–4 and pH greater than 7. However, at pH 4–7 it rapidly precipitates into an oligomeric β -sheet structure (Barrow and Zagorski, 1991). Nevertheless, the hydrophobic segment in the C-terminal domain of β A4 (residues 29–42) invariably adopts an oligomeric β -strand structure, independent of the pH or temperature, suggesting that this segment directs the complete protein folding (Barrow and Zagorski, 1991; Barrow et al., 1992). Thus, the first 28 residues, which are strongly dependent on factors such as the hydrophobicity and pH of the environment, enable β A4 to exist in different conformations (Table 1).

The change in structure from an α -helix to a β -pleated sheet corresponding with the change from acidic to midrange pH has been modeled (Zagorski and Barrow, 1992). The model is based on the presence of ionizable groups, such as the side chains of aspartic and glutamic acids, present in the conformational neighboring residues of the α -helix: amino acids 7, 11, and 22. These residues can ionize between pH 3 and pH 5; therefore, electrostatic repulsions between ionized carboxylates may disrupt the α -helix.

The hypothesis proposing that pH value is instrumental in β A4 deposition and senile plaque formation

TABLE 1. Structural characteristics of the β A4 peptides in different experimental conditions determined by various techniques

Fragment	Structure	Experimental condition	Methodology
1-28	β -Strand	Lyophilized peptides	X-ray diffraction ^a
1-28	α -Helix or random coil	pH 1-4 or pH > 7	Circular dichroism and bidimensional NMR ^{b,c}
1-28	β -Strand	pH 4-7	X-ray diffraction ^a
12-28	β -Strand	Lyophilized peptides	X-ray diffraction ^a
14-28			
1-4	α -Helix		Chou and Fasman (1978) analysis
6-8	β -Turn		
14-19	β -Strand		
17-21	α -Helix		
22-27	β -Turn		
29-42	β -Strand		
26-29	β -Turn	Analogue peptides with disulfide bridges	Limited proteolysis ^d
29-42	β -Strand	pH 1-10	Circular dichroism and bidimensional, NMR ^{b,c}
34-42	β -Strand	Lyophilized peptides and D ₂ O solution	X-ray diffraction and FTIR ^e
12-43	β -Strand	Lyophilized peptides	Infrared spectroscopy ^d

^a Gorevic et al., 1987.^b Barrow and Zagorski, 1991.^c Zagorski and Barrow, 1992.^d Hilbich et al., 1991.^e FTIR, Fourier transform infrared spectroscopy; Halverson et al., 1990.

is supported by the more acidic pH found in brains of AD patients (pH 6.6) compared with the neutral pH (7.1) of brains from patients who died suddenly with no brain disease (Yates et al., 1990). In this context, a number of mechanisms may produce localized pH fluctuation in vivo. For example, hypoxia (ischemia) of cerebral tissue may decrease brain pH from 7.1 to 6.0, and the reductions are even greater when ischemia is accompanied by hyperglycemia, shock, hypotension, or renal disorders, which are common in elderly people where the incidence of AD is greatest (Gilboe et al., 1986). Other mechanisms may involve local neurotransmitter alterations, contact with neutrophils and monocytes, or the presence of degenerating neurons.

β A4 STRUCTURE IN RELATION TO ITS TOPOGRAPHICAL DISTRIBUTION

The existence of β A4 in different states of aggregation related to the phase of formation of the amyloid plaque has been suggested by studies using various antibodies directed against different regions of the β A4 molecule (Spillantini et al., 1990). This, together with the pH and hydrophobicity effects on β A4 structure, suggests that β A4 exists in different conformational states according to its localization. Table 1 summarizes the secondary structures that may adopt the various fragments of β A4, depending of the environmental conditions. It is important to notice that when β A4 is in solution, the C-terminal domain (residues 29-42) always displays a β -strand conformation. There are still no conformational studies of this segment within the lipidic membrane. Notwithstanding, this segment is unlikely to display a β -strand structure within the

cell membrane. This hypothesis is based on the fact that in membranes only two secondary structures are possible: α -helix or β -sheet barrel (Jennings, 1989). As in the membrane, water molecules are not available to interact with polar groups of the polypeptide backbone; hydrogen bonds must be formed among the backbone atoms themselves. This results in the polypeptide chain folded into an ordered structure, α -helix or β -strand. In the case of α -helix, the hydrogen bonds can be formed within one helix, so that a single whole hydrophobic helix or any of several amphipathic helices may represent a stable structure in the membrane. In the case of β -strands, the hydrogen bonds must be formed between neighboring strands, leading to a closed structure, a β -barrel with a minimum of eight strands (Jähnig, 1990). Considering these findings, we propose that the β A4 segment within the membrane (amino acids 29-42) has an α -helix structure. It has been calculated that an α -helix needs 20 amino acids to span the plasma membrane and a β -sheet needs 10 (Jähnig, 1990). The transmembrane domain of APP is 23 amino acids long, thus making possible our suggestion. On the other hand, the amino acid sequence between residues 1 and 28 may be found in an α -helical or β -strand conformation, depending of the pH (Table 1). This sequence is likely to determine the existence of different conformational states of β A4.

β A4 occurs in different subcellular loci. First, β A4 is located in the plasma membrane as a moiety of APP. Then, after APP internalization, β A4 is found in lysosomal/endosomal structures. Finally, β A4 may be released to the extracellular space as a soluble entity, where later it can aggregate into amyloid fibrils. Some biochemical properties, such as pH, are different in

these subcellular regions, which could produce variations in the β A4 conformation, principally in its N-terminal domain. This fact could be very important in the understanding of the molecular basis of amyloid deposition.

CONCLUSIONS

The study of the structural features of β A4 peptide has improved our understanding of the pathogenesis of AD, including the factors that promote insolubilization of the peptide and subsequent fibril formation.

β A4 would contain two conformational domains: the C-terminal segment (amino acids 28–42) is incorporated within the plasma membrane and always exists in solution as a β -strand; the N-terminal domain adopts alternative secondary structures, depending on pH, hydrophobicity, and the presence of extracellular matrix components. With these alternative structures, β A4 can adopt two distinct conformations with different solubility properties, which could explain why the same amino acid sequence is found dissolved or in an aggregated form.

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A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors

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The amyloid proteins isolated from neuritic plaques and the cerebrovasculature of Alzheimer's disease are self-aggregating moieties termed A4 protein¹ and β -protein^{2,3}, respectively. A putative A4 amyloid precursor (herein termed A4₆₉₅) has been characterized by analysis of a human brain complementary DNA⁴. We report here the sequence of a closely related amyloid cDNA, A4₇₅₁, distinguished from A4₆₉₅ by the presence of a 168 base-pair (bp) sequence which adds 57 amino acids to, and removes one residue from, the predicted A4₆₉₅ protein. The peptide predicted from this insert is very similar to the Kunitz family of serine proteinase inhibitors. The two A4-specific messenger RNAs are differentially expressed: in a limited survey, A4₇₅₁ mRNA appears to be ubiquitous, whereas A4₆₉₅ mRNA has a restricted pattern of expression which includes cells from neuronal tissue. These data may have significant implications for understanding amyloid deposition in Alzheimer's disease.

Two full-length A4-specific cDNA clones were isolated from a λ gt10 library and were characterized (Fig. 2). Both cDNA clone sequences were identical to the A4₆₉₅ sequence⁴ except for a 168 bp insert (Fig. 2) resulting in an insertion of 57 amino acids and the removal of one residue of the predicted 695 amino-acid sequence of the A4₆₉₅ protein⁴. This 57 amino-acid sequence was compared to the protein sequence database of the Protein Identification Resource. The search revealed extensive similarity between the insert and the Kunitz family of low relative molecular mass serine proteinase inhibitors (Fig. 1): for the proteins compared in the figure the identity ranges from 33 to 48%. This similarity suggests that the A4₇₅₁ insert could act as a proteinase inhibitor.

To investigate the expression of A4₇₅₁ and A4₆₉₅ mRNAs, two

synthetic oligonucleotides were synthesized. One oligonucleotide corresponds to 60 bases of the 168-bp insert (insert probe) and the other oligonucleotide spans the insert point, possessing 15 bases on either side (junction probe). Under hybridization wash conditions favouring longer oligonucleotides the junction probe is stable when bound to A4₆₉₅ mRNA (30 bp perfect match), but unstable when bound to A4₇₅₁ mRNA (15 bp match). These probes were used to characterize the A4₆₉₅ and A4₇₅₁ cDNA representation in four cDNA libraries prepared from human RNA derived from a SV40-transformed fibroblast cell line, lymphocytes, and normal and Alzheimer's disease brain (see legend to Fig. 2). Insert-positive clones were identified in all libraries; however, only the brain libraries contained junction-positive clones. These results suggested that the expression of the A4-specific transcripts might be differentially regulated. We examined this possibility by characterizing steady-state mRNA levels in several different sources.

Polyadenylated RNA was prepared from human cultured cell lines and from human brain sections. The cultured cells used were HeLa, MRC5 and IMR-32 (see legend to Fig. 3). Tissue samples represented normal cerebellum, frontal and parietal cortex and frontal cortex from an Alzheimer's disease patient. An identical pair of Northern blots were prepared and probed with either the insert or junction oligonucleotide. Both blots were then stripped and reprobed with an actin cDNA probe as an internal control. The results of the Northern analysis (Fig. 3) reveal that each oligonucleotide specifically detects a 3.2–3.4 kilobase (kb) mRNA, consistent with the size of the mRNA encoding the putative A4 amyloid precursor^{4–7}. The junction probe, however, hybridizes to only a subset of the samples, those of neural origin (Fig. 3a, lanes 1, 6–9). No mRNA is detected in the HeLa or MRC5 samples using the junction probe even in the deliberately overexposed autoradiograph shown. In contrast, the insert probe detects sequences in all samples (Fig. 3b). The relative expression levels of the two mRNAs were also examined by RNA dot blot using total RNA prepared from several additional cell lines (P.G.-D. and P.P., data not shown). A human glioblastoma line (U-87-MG) and two human neuroblastoma lines (SK-N-MC and SK-N-SH) all contain mRNA species which hybridize to the insert probe, but none of these samples show significant hybridization to the junction probe above background levels (using MRC5 RNA as the negative control).

At least two types of A4-specific mRNA (and presumably protein) exist which are differentially regulated. We have also determined that the inserted sequence is entirely and exclusively encoded on a separate exon (J.M., F.F. & D.H. unpublished observation). From information on Alzheimer's disease amyloid

Protein	296	300	310	320	330	340	% Identity	Ref.
A4 ₇₅₁ Insert	EVQSEAEETGPRANISRVYFDVTEGKAAETFGGGGNGNINFDTEETCMAYGSSAI						-	-
Bovine pancreatic inhibition	DFCLEPPYTGPRKAEIIRVFNNAKAGLOOTFVYGGGPAKRNNKSAEDCKRTGGAI						47	24
Bovine serum inhibition	DFCLEPPYTGPRKAEIIRVFNNAKAGLOOTFVYGGGPAKRNNKSAEDCKRTGGAI						42	31
Bovine colostrum inhibition	DLOLPGARGPGKAAALLRFFDYDSTSHACEPTFGGGGNGNNDNFETTEMGLRICEPPO						42	32
Human ITI Domain I	DSQDLGYSAGPGYKMTSRFFYNGTSHAKETFLGGGNGNINFLSQKEGLQCTRTV						43	23
Domain II	AAQNLPIVIRGPRAFIQLWAFDAVKGRVUPFGGGGNGNINFLSQKEGLQCTRTV						46	
Bovine ITI Domain I	DSQDLGYSAGPGYKMTSRFFYNGTSHAKETFLGGGNGNINFLSQKEGLQCTRTV						33	24
Domain II	EAQNLPIVIRGPRAFIQLWAFDAVKGRVUPFGGGGNGNINFLSQKEGLQCTRTV						46	

Fig. 1 Comparison of the amino-acid sequences of the A4₇₅₁ insert with Kunitz proteinase inhibitors. The original computer search was conducted against the Protein Identification Resource database using the FASTP routine of Pearson and Lipman²². Only mammalian proteins are compared although homologous proteins which are members of this family have also been isolated from reptiles, molluscs and coelenterates (reviewed in ref. 23). ITI, inter- α trypsin inhibitor. Only the 57 residues that overlap with the A4₇₅₁ insert are displayed. Numbering is based on the A4₇₅₁ predicted protein sequence as defined in Fig. 1. The 13 residues boxed are invariant in the other members of this inhibitor family and are all conserved in the A4₇₅₁ insert. The percentage identity of each sequence with the A4₇₅₁ insert sequence is shown. Human and bovine ITI domain I is glycosylated at Asn-310.

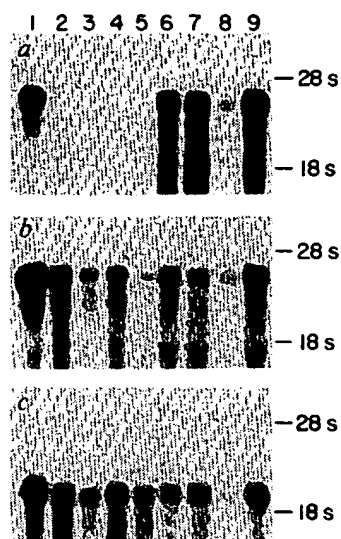


Fig. 3 Northern analysis of A4_{m95} and A4₇₅₁ mRNA species. Total or cytoplasmic RNA was isolated from human adult brain sections or cells propagated in culture. The cultured cells are HeLa, an epithelial-like cell derived from a cervical carcinoma; MRC5, a primary fibroblast line from fetal lung tissue; IMR-32, a mixed culture developed from a peripheral neuroblastoma with the predominant cell of neuroblast-like characteristics and the minor type resembling a hyaline fibroblast. All cell lines used are available from the American Type Culture Collection. Total RNA preparations from brains were isolated by the guanidine thiocyanate/LiCl method of Cathala *et al.*²⁴. A cytoplasmic fraction from confluent monolayers of HeLa and MRC5 cells was prepared by lysis of the cells in a hypotonic buffer of 10 mM Tris pH 7.5, 10 mM KCl, 1 mM MgCl₂, 1% Triton-X100, 0.1% sodium deoxycholate for 10 min at 4°C, homogenization in a Dounce with a 'B' pestle for 10 strokes, and pelleting the nuclei at 2,500g for 5 min. The supernatant was mixed with guanidine thiocyanate, and RNA was pelleted through CsCl by the method of Chirgwin *et al.*²⁵. Total RNA from the same cells was obtained by lysis of the cells directly into guanidine thiocyanate followed by CsCl fractionation as above. RNA (100–400 µg) was fractionated by oligo(dT) cellulose chromatography by the method of Aviv and Leder²⁶. Samples containing poly(A)⁺ RNA were divided into two aliquots, run on duplicate formaldehyde/1% agarose gels, and blot-transferred to nitrocellulose by the method of Thomas²⁷. Nitrocellulose filters were hybridized, either to the junction oligonucleotide (5'-(TGCTGTTGTAGGAACCTCGAACACCTCTT-3') or the insert oligonucleotide

(5'-CGCCGTAAGAATGGGGCACACTTCCCTTCAGTCA
CATCAAGTACCAGCGGGAGATCA-3')

which had been labelled with a 15–30 nucleotide-long homopolymeric tail using [α -³²P]dCTP and terminal deoxynucleotidyl transferase as previously described²⁸. The buffers used for hybridization contained 10% dextran sulphate, 5× Denhardt's reagent, 50 mM sodium phosphate (pH 6.7), with SSC and formamide concentrations dependent on the probe used—junction oligonucleotide: 20% formamide, 6× SSC; insert oligonucleotide: 34% formamide, 4× SSC; actin: 50% formamide, 4× SSC. Blots were washed to a final stringency of 1× SSC at 55°C, and exposed to Kodak X-AR film for 3 days. Both blots were then stripped and rehybridized to a human β -actin cDNA insert²⁹ which had been labelled by nick-translation with [α -³²P]dCTP³⁰. Re-exposure (6 h) of the filter used in *a* is shown in *c*; re-exposure of filter shown in *b* is very similar and is not presented. The RNA samples displayed are: Lane 1, total IMR-32; 2, total MRC5; 3, total HeLa; 4, cytoplasmic MRC5; 5, cytoplasmic HeLa; 6, normal cerebellum; 7, normal frontal cortex; 8, AD frontal cortex; 9, normal parietal cortex. *a*, RNAs hybridized with the junction oligonucleotide; *b*, RNAs hybridized with the insert oligonucleotide; and *c*, RNAs hybridized with human β -actin cDNA. Ribosomal RNAs used as internal size markers are shown. The normal brain samples were obtained from two different individuals, 98 years of age (frontal cortex and cerebellum samples) and 60 years of age (parietal cortex sample), both lacking clinical signs of dementia. The Alzheimer's disease sample was obtained from a 97-year-old patient displaying overt clinical indications of the disease. The tissue samples were obtained 4, 14 and 5 h post-mortem from the 98, 60 and 97-year-old individuals, respectively. Because different amounts of RNA may be contained in each sample, determination of the relative amounts of each A4-specific species in the different samples is not possible.

ing of the distribution and expression levels of these two A4-specific mRNAs in normal and in pathological conditions such as Alzheimer's disease. The single Alzheimer's disease cortex sample examined here shows no striking differences in the relative amounts of each mRNA compared to the normal cortex samples.

The similarity of the 57-amino-acid insert to a family of proteinase inhibitors suggests that it may have a similar function. The biosynthesis and physiological role of this proteinase inhibitor family are not well understood. Bovine pancreatic trypsin inhibitor is derived from a larger precursor¹⁴ and the other members of the family may be as well. In each case, the functional inhibitor is ~57 amino acids long or a dimer of two such units. The precursor of the human inter- α -trypsin inhibitor may be functionally active¹⁵. Similarly, the entire A4₇₅₁ protein might function as a proteinase inhibitor or the putative inhibitor domain may be proteolytically excised and function independently. The physiological role of other serine proteinase inhibitors is the regulation of a single proteinase¹⁶. The analysis of the physiological function of the A4₇₅₁ insert and the identification of its *in vivo* target must await the production of the pure protein.

We thank D. Wolf for the SV40-transformed fibroblast library, E. Stoelting for photographic work, C. Boyle and L. Brown for manuscript preparation, C. Bryant and T. Scheuermann for technical assistance and J. Feldman and L. Berg for their efforts in the computer facility. This work was supported by California Biotechnology, Inc. (B.C., P.P., P.G.-D., J.M., J.S., D.H., B.G., F.F.), by the Hartford and McKnight Foundations (I.L.) and the NIH (I.L., K.D., W.W.). The sequence data in this publication have been submitted to the EMBL/GenBank Libraries under the accession number Y00297.

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or even increased, in AD-affected frontal cortex. Conversely, the APP mRNA lacking HL124i or the specific cells containing this species may be severely reduced or eliminated in AD.

It is possible that the potential protease inhibitor domain encoded by HL124i alters the metabolism of APP, perhaps protecting the molecule from degradation by certain serine proteases. Kunitz-type inhibitors are specific for serine proteases such as trypsin, chymotrypsin, elastase, plasmin and cathepsin G (refs 16, 17). Whereas these enzymes could be inhibited by the HL124i domain in APP, other classes of proteases would presumably be refractory to its effects. Thus, the proteolytic intermediates resulting from the metabolism of the APP molecule could differ appreciably, depending on the presence or absence of the HL124i domain. Like human ITI, this domain could have anti-proteolytic activity both in the intact and excised state^{18,19}, giving APP the ability to inhibit the degradation of other proteins. It should be noted that the principal component of the vascular amyloid in the Icelandic form of hereditary cerebral amyloidosis is a variant of cystatin, a cysteine protease inhibitor²⁰. Alpha-1-antichymotrypsin, a serine protease inhibitor distinct from the Kunitz family, is a component of amyloid plaques in AD (C. Abraham, D. J. Selkoe and H. Potter, *Cell*, in the press). As the physiological function of Kunitz-type inhibitors is not understood²¹, HL124i could possibly provide the amyloid precursor with a new function unrelated to protease inhibition.

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Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity

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Alzheimer's disease¹ is characterized by cerebral deposits of amyloid β -protein (AP) as senile plaque core and vascular amyloid²⁻⁶, and a complementary DNA encoding a precursor of this protein (APP) has been cloned from human brain⁷⁻¹¹. From a cDNA library of a human glioblastoma cell line, we have isolated a cDNA identical to that previously reported, together with a new cDNA which contains a 225-nucleotide insert. The sequence of the 56 amino acids at the N-terminal of the protein deduced from this insert is highly homologous to the basic trypsin inhibitor family¹², and the lysate from COS-1 cells transfected with the longer APP cDNA showed an increased inhibition of trypsin activity. Partial sequencing of the genomic DNA encoding APP showed that the 225 nucleotides are located in two exons. At least three messenger RNA species, apparently transcribed from a single APP gene by alternative splicing, were found in human brain. We suggest that protease inhibition by the longer APP(s) could be related to aberrant APP catabolism.

We first screened various cell lines by RNA-blot hybridization using a synthetic oligonucleotide probe (AM-1, Fig. 1a), and found APP mRNA to be expressed strongly by human glioblastoma (ATCC HTB14, 16, 17), neuroblastoma (ATCC HTB10, 11) and neuroglioma (ATCC HTB148) cell lines, but only weakly

by hepatoma (HepG2), monocytic (THP-1) and lymphoblastoid (RPMI1788) cell lines (data not shown). Expression by glioblastoma ATCC HTB14 was strongest, and this cell line was therefore chosen for the cDNA cloning.

The cDNA was cloned in two separate fragments for efficiency, the *Bam*HI fragment (5'piece) and *Bam*HI-*Hind*III fragment (3'piece), as shown in Fig. 1a. Complete nucleotide sequencing of the *Bam*HI-*Hind*III fragment harboured by one clone showed it to be identical to that in the corresponding part of the reported APP cDNA (ref. 7).

Two species of the *Bam*HI fragment were isolated using AM-3 as hybridization probe, from a library prepared with AM-1 as primer. One was about 1.4 kilobases (kb) long and the other about 1.6 kb. The nucleotide sequence of the shorter cDNA (later ligated to form full-size cDNA pAPP695, coding for the 695-amino-acid peptide APP695) was identical to that of the 1,376-base-pair (bp) *Bam*HI fragment of the reported APP cDNA (ref. 7). Sequencing of the longer cDNA (later ligated to form full-size cDNA pAPP770, coding for the 770-amino-acid peptide APP770) showed that it differed only in the presence of a 225-bp insert between nucleotides (nt) 865 and 866 of pAPP695, as numbered from the first base of the putative translation-initiating codon. The amino-acid sequence deduced from the nucleotide sequence of the longer cDNA thus includes a novel 75-amino-acid insert in the highly acidic region of the putative extracellular domain⁷ of the APP (Fig. 1b), with valine, amino acid 289 of the original peptide, replaced by glutamic acid and leucine at respective ends of the insert (Fig. 1c).

The novel sequence of 76 amino acids was screened against the NBRF data base, when we found that its N-terminal 56-amino-acid sequence showed a strong similarity to the basic trypsin inhibitor family (Kunitz type), see Fig. 2 (ref. 12). The spacing of its six cysteines is consistent with the spacing found in the minikringle structure common to this family. Extensive congruency with the highly conserved region of this family (underlined in Fig. 2) is also seen, including the presence of a

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Table 1 Relative trypsin activity in COS-1 cells transfected with APP cDNAs (mean \pm s.e.m.)

Plasmid	None	pSVMT-APP695	pSVMT-APP770
run 1	100.0 \pm 15.0	94.0 \pm 12.4	29.0 \pm 15.9†
run 2	100.0 \pm 10.4	101.0 \pm 5.5	36.8 \pm 23.5*

The average value of "none" is set as 100%. Lysate of mock-transfected cells intrinsically showed 47–58% inhibition compared with lysate-free control. *Bam*HI (partial)-*Hind*III fragments of pAPP695 and pAPP770 were inserted into a mammalian expression vector between a mouse metallothionein-I promoter and an SV40 polyadenylation sequence. The plasmid (10 μ g) was transfected into 4×10^6 COS-1 cells by electroporation (3 μ F, 400V, 1.0–1.3 ms, twice, with 30 s interval). The transfected cells were cultured in Dulbecco's modified minimum essential medium with 10% fetal calf serum, and the medium changed after 24 h. Cells were collected, 41 h later and homogenized in extraction buffer (0.1M triethanolamine, 0.3M NaCl, 0.01M CaCl_2 , pH 7.8). Inhibition of trypsin activity was measured as described^{21,22} with modification. Cell lysate containing 100 μ g protein was incubated with trypsin for 5 min, and the trypsin activity measured using *N*- α -benzoyl-DL-arginine-7-amino-4-methyl-coumarine (Sigma) as a fluorogenic substrate.

* $P < 0.05$, † $P < 0.01$, Student *t*-test.

basic amino acid (denoted by an asterisk in Fig. 2) in the active site^{13,14}.

In Southern blot analysis of *Bam*HI-digested lymphocyte DNA from one normal individual and eight Alzheimer's disease patients with a 212-bp *Taq*I(nt 862)–*Ava*I(nt 1,073) fragment of pAPP770 (Fig. 1c) as hybridization probe, only a 6.6-kb band was commonly observed, suggesting that the 225-bp insert exists as a single copy, and that there is no significant difference among these genomic DNAs (data not shown). Genomic DNA clones containing the region of the 225-bp insert were isolated from the human leucocyte DNA library (Clontech, USA) using the 212-bp *Taq*I–*Ava*I fragment as probe. In the genomic DNA, the 225-bp sequence was located in a 168-bp exon and a 57-bp exon, separated by an intron of ~ 3 kb, with both exons flanked by intron-exon consensus sequences¹⁵. The 168-bp exon (tentatively designated I, among exons H, I, J and K of this region, as defined in Fig. 3a) corresponds to nt 866 to 1,033 of pAPP770, and the 57-bp exon (J) to nt 1,034 to 1,090. Exon I encodes the highly conserved region of the protease inhibitor family.

In vivo expression of the corresponding mRNA species was investigated by RNA-blot hybridization using 24-mer probes AM-11, AM-12, AM-13 and AM-14, complementary to the H-K, H-I, I-K and J-K junction sequences respectively (Fig. 3a). The results show that mRNA species with the H-I and the H-K sequences were both substantially expressed in fetal brain, whereas the mRNA with the H-I sequence was predominant in adult hippocampus. Comparison of mRNA species having the I-K and the J-K sequences shows that both are expressed in similar amounts in fetal brain, but in adult hippocampus the I-K sequence was expressed more than the J-K (Fig. 3b). These results indicate that at least three species of mRNA are expressed *in vivo*, apparently as a result of alternative splicing. In poly(A)⁺ RNA from HTB14 cell, the mRNA species with the I-K and J-K sequences predominated over that with the H-K sequence.

Greatly increased inhibition of trypsin activity was observed with APP770. Plasmids pSVMT-APP695 and pSVMT-APP770, expressing full-length APP695 and APP770, respectively, under the control of mouse metallothionein-I promoter, were constructed and transfected into COS-1 cells (legend to Table 1). The lysate of the cells transfected with pSVMT-APP770 inhibited trypsin activity against a synthetic substrate more strongly than the lysate of either mock-transfected cells or cells transfected with pSVMT-APP695 (Table 1).

It has recently been shown that the APP gene is not duplicated in Alzheimer's disease (both familial and sporadic) (refs. 16–18),

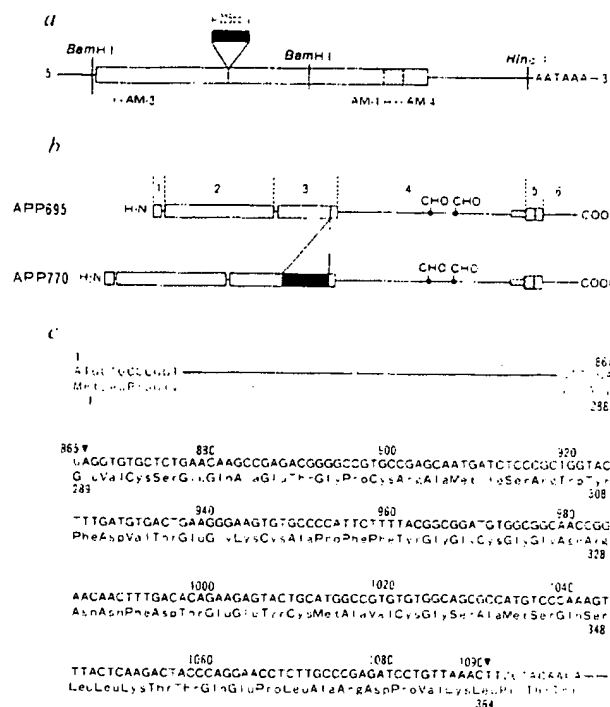


Fig. 1 APP cDNAs (pAPP695 and pAPP770), their predicted proteins, and the nucleotide sequence and deduced amino-acid sequence of the novel insert in pAPP770. *a*, Schematic representation of APP cDNAs. Putative coding region of APP cDNA (ref. 7) in open box; a novel 225-bp insert of pAPP770 in closed box; region coding for peptide of amyloid deposit in hatched box. Positions of oligonucleotide probes AM-3, 4 and primer AM-1 are indicated. *b*, Proposed domain structure of APP695 (ref. 7) and APP770. Domains: 1, signal sequence; 2, cysteine-rich region; 3, highly-negatively-charged region, with novel insert (closed box) in APP770; 4, *N*-glycosylation-site region; 5, transmembrane segment; 6, cytoplasmic domain. Hatched box, amyloid deposited in the brain. *c*, Nucleotide sequence and predicted amino-acid sequence of the novel insert of pAPP770. Nucleotide residues are numbered as described in the text. The novel insert (gothic) begins at nt 866 and ends at nt 1,090 (between arrowheads). The deduced amino acid sequence is numbered beginning with the amino-terminal methionine. The amino acid residues absent in APP695 are numbers 289–364 (gothic).

Methods. Glioblastoma HTB14 was purchased from American Type Culture Collection (ATCC) and cultured as recommended by ATCC. Total RNA was extracted²³ from $\sim 5 \times 10^6$ cells. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography. (1) Cloning of 3' piece of APP cDNA. Double-stranded cDNA was prepared by oligo(dT) priming using 10 μ g poly(A)⁺ RNA and digested with *Bam*HI and *Hind*III. Fragments of 1–2 kb were isolated by agarose gel electrophoresis and ligated into *Bam*HI–*Hind*III-digested pUC19, followed by transformation into *Escherichia coli* MC1061. Transformants (3.5×10^4) were screened with oligonucleotide probe AM-4 (5'-ACCATGAGTCCAATGATTGCAC-3'). 200 positive clones were obtained, of which 18 were subjected to restriction-site analysis with identical results. The sequence of one clone was determined by the dideoxy method^{24,25}. (2) Cloning of 5' piece of APP cDNA. Double-stranded cDNA was synthesized using oligonucleotide primer AM-1 (5'-CTTCATATCCTGAGTCATGTCG-3') and digested with *Bam*HI. Fragments of 0.7–2 kb were isolated and ligated into *Bam*HI-digested pUC19. Transformants (9×10^3) were screened with AM-3 probe (5'-CATTCATGTGTCATGTTCACTCTG-3') and 20 positive clones were obtained. One of 12 arbitrarily chosen clones had a 1.4-kb insert, as predicted from the reported sequence⁷. The other 11 clones had 1.6-kb inserts. The nucleotide sequence of the 1.4-kb insert and of the 1.6-kb insert was determined. Full-size APP cDNAs, pAPP695 and pAPP770, were constructed by ligation of the 1.4-kb and the 1.6-kb *Bam*HI fragments respectively to the 3' *Bam*HI–*Hind*III fragment.

Fig. 2 Alignment of the N-terminal 56 amino acids of the deduced novel 76-amino-acid insert sequence in APP770 with the known basic trypsin inhibitor family. The residues homologous to the highly conserved sequence of this family are underlined. The basic amino acid residue (Arg) in the active site is indicated by an asterisk. Residues identical to APP770 are boxed. To the left are given the residue numbers of the first aligned amino acid. Proteins: BPTI, bovine basic pancreatic trypsin inhibitor precursor; BIATI, bovine inter- α -trypsin inhibitor; HIATI, human inter- α -trypsin inhibitor; SA5II, sea anemone proteinase inhibitor 5II (ref. 13); VBPIII, Russell's viper venom basic protease inhibitor II

Protein (Number)	250	300	350	400	450
APP770 289	EVCSEQAETGPCRAM	SRWYFDVTEGKCAPFFYGGCGGNRRNF	EDTEECMAVCGSA		
BPTI 17	DFCLEPPYTGPKAR	IRFYNAKAGLCQTFVYGGCRKRNNF	KSIEDCMR	CGG	
BIATI 59	EACNLPVVGPCRAF	ILQLWAFDAVKGKCVFESYGGCGNGNKFYSQKECKE	YDGP		
HIATI 80	AACNLPVIRGPCRAF	ILQLWAFDAVKGKCVLFYGGCGNGNKFYSQKECKE	YDGP		
SA5II 3	GDCELPKVVGPCRAR	FRPRVYVNSSSKPKCKFIYGGCGGNANNF	ETLEEC	KVCG	
VBPIII 5	FCNLAPESGRCRG	HLRRIYVNLSEKCKVFFYGGCGGNANNF	ETREDE	RET	CGG

Fig. 3 Expression of APP mRNA in human brain. *a*, Schematic representation of the synthetic oligonucleotide probes (24-mers) designed to detect alternatively spliced APP mRNA species. Regions H, I, J and K correspond to the exons tentatively designated by the same letters (see text). AM-11 (5'-CTGTTGTAG-GAAGTGAACACCT-3') is complementary to the H-K junction; AM-12 (5'-CAGAGCACCTCTCGAACCACCT-3') to the H-I junction; AM-13 (5'-CTGTTGTAGGAATGGCGCTGC-CAC-3') to the I-K junction; AM-14 (5'-CTGTTGTAGGA-AGTTTAACAGGAT-3') to the J-K junction; nt, nucleotide number. *b*, Northern blot analysis of human brain poly(A)⁺ RNA. Lanes 1, 4, 7 and 10: poly(A)⁺ RNA from adult brain (70-year-old male, hippocampus); lanes 2, 5, 8 and 11: from fetal brain (10-week-old aborted fetus); lanes 3, 6, 9 and 12: from glioblastoma HTB14. Hybridization probes are indicated. *c*, Southern hybridization to determine the specificity of probes. Lanes contain BamHI-digested pSVMT-APP695 (lanes 13, 15, 17 and 20), pSVMT-APP751 (lanes 18 and 21) or pSVMT-APP770 (lanes 14, 16, 19 and 22). Hybridization probes are indicated.

Methods. Total cellular RNAs were prepared from fetal brain and HTB14 cells by the guanidium/CsCl method²³ and poly(A)⁺ RNAs were isolated by oligo(dT)-cellulose chromatography. Poly(A)⁺ RNA from adult brain was purchased from Clontech Inc. Glyoxal-denatured poly(A)⁺ RNA (2.5 µg per lane) was fractionated on 1% agarose gel, run in 10mM sodium phosphate (pH 7.0) (ref. 26) and subsequently transferred to Zeta Probe (Bio-Rad). An RNA ladder (BRL) was used as size marker. For Southern blot analysis, pSVMT-APP751 containing the H-I-K sequence was constructed from pSVMT-APP770 using AM-13 as delecter²⁷. BamHI-digested pSVMT-APP695, pSVMT-APP751 or pSVMT-APP770 were transferred to Zeta Probe. Each filter was hybridized with ³²P-labelled oligonucleotide probe in 5×SSC, 25mM sodium phosphate (pH 7.0), 5×Denhardt's solution, 1% glycine, 0.1% SDS at 55 °C for 2 h. The filters were washed at 55 °C in 6×SSC, 0.1% SDS.

and that the APP gene is not tightly linked to the familial Alzheimer's disease (FAD) locus^{19,20}. Possibly then, the FAD gene product could affect alternative splicing of the APP gene. Although no serine protease(s) which would be inhibited by APP770 or its additional fragments has yet been identified, we suggest that protease inhibition might be involved in the aberrant processing of APP, resulting in the deposit of amyloid β -protein.

The new species of APP cDNA could therefore be important in the investigation of the pathogenesis of Alzheimer's disease and Down's syndrome. During the preparation of this manuscript we learned that two other groups have isolated cDNA clones encoding APP with a sequence homologous to the trypsin inhibitor.

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Figure 3

Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease

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Amyloid B-protein/amyloid A4 is a peptide present in the neuritic plaques, neurofibrillary tangles and cerebrovascular deposits in patients with Alzheimer's disease and Down's syndrome (trisomy 21)¹⁻⁴ and may be involved in the pathogenesis of Alzheimer's disease⁵. Recent molecular genetic studies have indicated that amyloid protein is encoded as part of a larger protein by a gene on human chromosome 21 (refs 6-9). The amyloid protein precursor (APP) gene is expressed in brain and in several peripheral tissues, but the specific biochemical events leading to deposition of amyloid are not known. We have now screened complementary DNA libraries constructed from peripheral tissues to determine whether the messenger RNA encoding APP in these tissues is identical to that expressed in brain, and we identify a second APP mRNA that encodes an additional internal domain with a sequence characteristic of a Kunitz-type serine protease inhibitor. The alternative APP mRNA is present in both brain and peripheral tissues of normal individuals and those with Alzheimer's disease, but its pattern of expression differs from that of the previously reported APP mRNA.

We screened a cDNA library constructed from mRNA of the human promyelocytic leukaemia cell line HL60 to find clones hybridizing with FB68L, a fetal brain cDNA for the 3' portion of the APP message (Fig. 1). One of the clones obtained, HL12, has additional restriction sites not found in a previously reported APP cDNA (ref. 7). HL12 contains a sequence identical to that of the previously reported cDNA, except for an insertion of 168 base pairs (bp) of DNA between positions 865 and 866. This novel DNA sequence, designated HL124i, occurs within the codon for Val289 of APP. The HL124i insert preserves the reading frame of the APP message, contains no termination codons, and would introduce 56 amino acids C-terminal to Arg288 of the APP protein, converting Val289 into an isoleucine. The HL124i segment recognizes a previously described¹⁰ *BanI* restriction fragment length polymorphism (RFLP) that is not detected by any other portion of HL12, but demonstrates tight genetic linkage to the other RFLPs at the APP locus on human chromosome 21. Somatic cell hybrid analysis confirms that both FB68L and HL124i map to chromosome 21q11.2-q21 (data not shown). Figure 1 indicates where two potential splicing events could generate a message containing the novel sequence, assuming that the HL124i segment is encoded as one continuous exon in a single APP gene.

In a search of the protein sequence database of the National Biomedical Research Foundation (Release no. 13), we found a

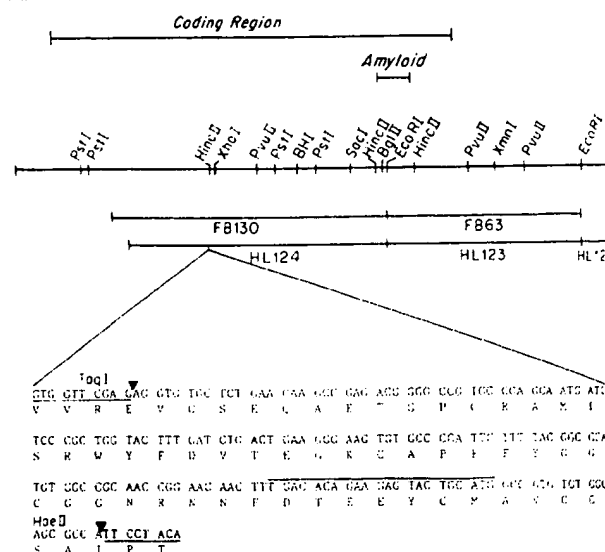


Fig. 1 Sequence analysis of the HL124i segment and restriction map of the full-length APP cDNA. HL12 consists of three *EcoRI* fragments which were subcloned into pUC18 and designated HL122, HL123 and HL124. The sequence of the HL124i segment begins 3' to bp 865, interrupting Val289 in the APP cDNA sequence reported by Kang *et al.*⁷ The HL124i sequence is not present in the corresponding fetal brain cDNA, FB130. Sequences present in both HL124 and FB130 which flank the 3' and 5' ends of HL124i are underlined. Arrows indicate potential splice junction sites in HL124. The 22-bp region to which an anti-sense oligonucleotide was constructed for Northern blot analyses is overlined. The *TaqI* and *HaeIII* sites used to excise the HL124i segment from HL124 for use as a probe in Southern blot analyses are also shown. The 3' cDNA FB63 is identical to FB68L. Sequencing was performed by the dideoxy method²¹ directly on pUC18 subclones using a modified protocol from New England Biolabs and a modified T7 DNA polymerase²² on M13 subclones.

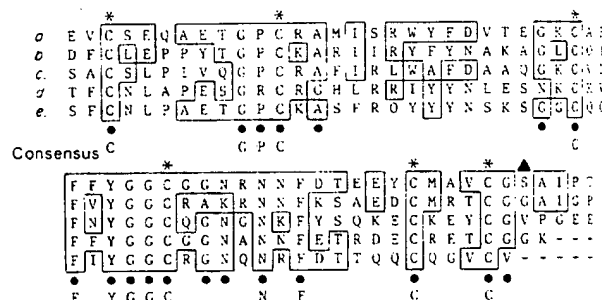


Fig. 2 Similarity between the protein encoded by HL124i and the Kunitz domain II regions of four basic protease inhibitors. Amino acid identities are boxed. Asterisks denote the six conserved cysteine residues. Consensus amino acids present in all five sequences are indicated below. Filled circles indicate residues present in four out of five of the sequences. A filled triangle marks the amino acid residue at which the Kunitz domain (II) terminates. The sequences are of the following proteins: a, HL124i encoded Domain in APP; b, basic protease inhibitor precursor-bovine (fragment)²⁴; c, human serum inter- α -trypsin inhibitor²⁵; d, venom basic protease inhibitor II (viper)²⁶; e, Isoinhibitor K (BPI type) (edible snail)²⁷.

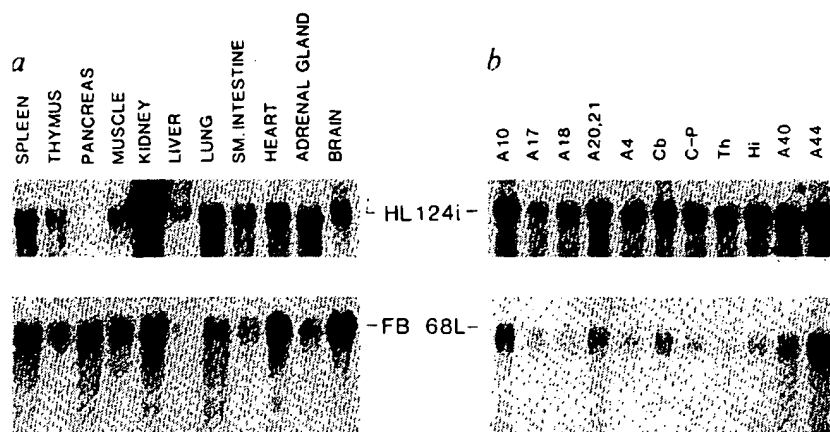
group of 17 polypeptides with sequences similar to that encoded by HL124i. All are members of a family of Kunitz-type serine protease inhibitors^{11,12}. Figure 2 shows the four inhibitors most similar to the predicted HL124i amino acid sequence: these include the C-terminal domain (II) of human serum inter- α -trypsin inhibitor (ITI) with 50% similarity. Human ITI consists of two connected domains embedded in a larger precursor²⁵. Domain II has a selective affinity for trypsin¹⁴; domain I, which

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Fig. 3 Comparison of FB68L- and HL124i-hybridizing mRNAs. Hybridizations of HL124i and FB68L to RNA (20 µg) from human 20-22-week fetal tissues. Fetal tissue was obtained from mid-trimester elective abortions under protocols approved by the institutional review board at Brigham and Women's Hospital. The blot to which HL124i was hybridized was equivalent to that used in the FB68L hybridization, except that the former lacked pancreatic RNA; a space has been inserted in the HL124i blot above the pancreatic lane of the FB68L blot. The minor hybridization band above and the APP band, seen in the liver lane, represents non-specific binding of the oligonucleotide probe to 28S ribosomal RNA. *b*, Hybridization of HL124i and FB68L to RNA (10 µg) from adult human brain subregions: A10, frontal pole of the cortex; A17, striate cortex; A18, extrastriate cortex; A20, 21, temporal association cortex; A4, motor cortex; Cb, cerebellum; C-P, caudate-putamen; Th, thalamus-ventral posterolateral nucleus; Hi, hippocampus; A40, posterior perisylvian cortex-supramarginal gyri; A44, anterior perisylvian cortex-opercular gyri. The two autoradiograms are the result of two independent hybridizations with the same filter. Methods of RNA isolation and hybridization with FB68L have been previously described⁶. Hybridizations with the oligonucleotide were carried out in 5 × SSC and 50% formamide at 30 °C, followed by three 30 min. washes in 3 × . 2 × and 1 × SSC at room temperature.



does not inhibit trypsin but may interact with other serine proteases¹⁴, shows less similarity (44%) to the predicted HL124i sequence. The nucleotide sequence for domain II (ref. 13) shares 57% homology over 171 bp with HL124i. The polypeptide encoded by HL124i contains many amino acids in consensus positions characteristic of a functional Kunitz domain, including the six cysteine residues that are conserved among Kunitz-type protease inhibitors from cow, snake, turtle and edible snail (N. Ramesh, personal communication)¹⁵.

The cDNAs with and without HL124i should represent different messages generated from the APP gene. We have previously demonstrated⁶ that the fetal brain clone FB68L, which recognizes both forms, hybridizes to 3.4-3.6-kilobase (kb) mRNA species in various human fetal tissues. We have compared this pattern with the hybridization of a 22-base oligonucleotide that should recognize only HL124i-containing mRNAs (Fig. 1). This oligonucleotide also detects 3.4-3.6-kb mRNA species, but with striking differences in tissue distribution. Hybridization with FB68L gives relatively high signal intensity in human fetal brain, heart, and spleen and a barely detectable signal in liver, whereas the HL124i oligonucleotide detects the largest amount of mRNA in kidney and uniform levels among the other tissues, including liver (Fig. 3a). Thus, the relative proportion of these alternative forms of the APP message varies from tissue to tissue, reflecting possible differential regulation of RNA processing or stability. In adult human brain, total APP message detected by FB68L is concentrated in association cortex, specifically in Brodmann areas A10, 44, 20/21, and 40 (ref. 6). But hybridization with the HL124i-specific oligonucleotide shows that the APP transcript containing HL124i is distributed homogeneously among the different brain regions. By subtraction, this difference implies that the APP mRNA lacking the HL124i sequence is selectively expressed in association areas of the brain.

The hybridization of the two probes to mRNA from brains of normal individuals, patients with Down's syndrome (DS), and victims of Alzheimer's disease (AD) is shown in Fig. 4. Relative amounts of mRNA were controlled by hybridization with a cDNA for the microtubule-associated protein, tau. With both APP probes, mRNA from DS fetal brain gives a clear increase in signal intensity over normal. Hybridization with FB68L produces a stronger signal in fetal brain mRNA than in mRNA from adult brain⁶, but the HL124i oligonucleotide detects more mRNA in adult brain regions, suggesting that the

APP mRNA lacking HL124i is selectively expressed to a greater extent in the fetal brain. The total APP mRNA labelled by FB68L and the HL124i-containing component do not differ substantially in the adult cerebellum from patients with AD controls. The ratio of FB68L:HL124i signal, however, is lower in the cerebellum from the AD patient. This difference is more dramatic in the adult frontal cortex. Although the signal produced by FB68L is significantly reduced from AD to normal, the HL124i-containing mRNA seems relatively preserved. The mRNA from affected frontal cortex also shows a substantial loss of tau signal, and displays weaker than normal signal for a variety of neuronal and non-neuronal cDNA probes (R.L.N., unpublished data). Therefore, either the HL124i-containing APP mRNA itself, or the cells expressing it may be selectively spared.



Fig. 4 Northern blot of HL124i and FB68L hybridizations to total RNA (25 µg) from 19-week normal (lane a) and trisomy 21 (lane b) brains, adult normal (lane c) and AD (lane d) cerebellum and adult normal (lane e) and AD (lane f) frontal cortex. Fetal tissue was obtained from an abortion with a diagnosis of DS and from an age-matched normal abortion. Adult tissue was obtained from autopsy brains of a case of histologically confirmed AD and from an individual without dementing illness. Control hybridization with a cDNA for the microtubule-associated protein tau²⁸ is shown above the results for HL124i and FB68L. The three autoradiograms are the results of independent hybridizations with the same filter.

or even increased, in AD-affected frontal cortex. Conversely, the APP mRNA lacking HL124i or the specific cells containing this species may be severely reduced or eliminated in AD.

It is possible that the potential protease inhibitor domain encoded by HL124i alters the metabolism of APP, perhaps protecting the molecule from degradation by certain serine proteases. Kunitz-type inhibitors are specific for serine proteases such as trypsin, chymotrypsin, elastase, plasmin and cathepsin G (refs 16, 17). Whereas these enzymes could be inhibited by the HL124i domain in APP, other classes of proteases would presumably be refractory to its effects. Thus, the proteolytic intermediates resulting from the metabolism of the APP molecule could differ appreciably, depending on the presence or absence of the HL124i domain. Like human IT1, this domain could have anti-proteolytic activity both in the intact and excised state^{18,19}, giving APP the ability to inhibit the degradation of other proteins. It should be noted that the principal component of the vascular amyloid in the Icelandic form of hereditary cerebral amyloidosis is a variant of cystatin, a cysteine protease inhibitor²⁰. Alpha-1-antichymotrypsin, a serine protease inhibitor distinct from the Kunitz family, is a component of amyloid plaques in AD (C. Abraham, D. J. Selkoe and H. Potter, *Cell*, in the press). As the physiological function of Kunitz-type inhibitors is not understood²¹, HL124i could possibly provide the amyloid precursor with a new function unrelated to protease inhibition.

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Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity

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Alzheimer's disease¹ is characterized by cerebral deposits of amyloid β -protein (AP) as senile plaque core and vascular amyloid²⁻⁶, and a complementary DNA encoding a precursor of this protein (APP) has been cloned from human brain⁷⁻¹¹. From a cDNA library of a human glioblastoma cell line, we have isolated a cDNA identical to that previously reported, together with a new cDNA which contains a 225-nucleotide insert. The sequence of the 56 amino acids at the N-terminal of the protein deduced from this insert is highly homologous to the basic trypsin inhibitor family¹², and the lysate from COS-1 cells transfected with the longer APP cDNA showed an increased inhibition of trypsin activity. Partial sequencing of the genomic DNA encoding APP showed that the 225 nucleotides are located in two exons. At least three messenger RNA species, apparently transcribed from a single APP gene by alternative splicing, were found in human brain. We suggest that protease inhibition by the longer APP(s) could be related to aberrant APP catabolism.

We first screened various cell lines by RNA-blot hybridization using a synthetic oligonucleotide probe (AM-1, Fig. 1a), and found APP mRNA to be expressed strongly by human glioblastoma (ATCC HTB14, 16, 17), neuroblastoma (ATCC HTB10, 11) and neuroglioma (ATCC HTB148) cell lines, but only weakly

by hepatoma (HepG2), monocytic (THP-1) and lymphoblastoid (RPMI1788) cell lines (data not shown). Expression by glioblastoma ATCC HTB14 was strongest, and this cell line was therefore chosen for the cDNA cloning.

The cDNA was cloned in two separate fragments for efficiency, the *Bam*HI fragment (5'piece) and *Bam*HI-*Hind*III fragment (3'piece), as shown in Fig. 1a. Complete nucleotide sequencing of the *Bam*HI-*Hind*III fragment harboured by one clone showed it to be identical to that in the corresponding part of the reported APP cDNA (ref. 7).

Two species of the *Bam*HI fragment were isolated using AM-1 as hybridization probe, from a library prepared with AM-1 as primer. One was about 1.4 kilobases (kb) long and the other about 1.6 kb. The nucleotide sequence of the shorter cDNA (later ligated to form full-size cDNA pAPP695, coding for the 695-amino-acid peptide APP695) was identical to that of the 1,376-base-pair (bp) *Bam*HI fragment of the reported APP cDNA (ref. 7). Sequencing of the longer cDNA (later ligated to form full-size cDNA pAPP770, coding for the 770-amino-acid peptide APP770) showed that it differed only in the presence of a 225-bp insert between nucleotides (nt) 865 and 866 of pAPP695, as numbered from the first base of the putative translation-initiating codon. The amino-acid sequence deduced from the nucleotide sequence of the longer cDNA thus includes a novel 75-amino-acid insert in the highly acidic region of the putative extracellular domain⁷ of the APP (Fig. 1b), with valine, amino acid 289 of the original peptide, replaced by glutamic acid and leucine at respective ends of the insert (Fig. 1c).

The novel sequence of 76 amino acids was screened against the NBRF data base, when we found that its N-terminal 56-amino-acid sequence showed a strong similarity to the basic trypsin inhibitor family (Kunitz type), see Fig. 2 (ref. 12). The spacing of its six cysteines is consistent with the spacing found in the minikrinle structure common to this family. Extensive congruency with the highly conserved region of this family (underlined in Fig. 2) is also seen, including the presence of a

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CLUSTAL W (1.74) multiple sequence alignment

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sp|P05067|A4_HUMAN      MLPGLALLLLAAWTARA  LEVPTDGNAGLLAEPQIAMFCGRLNMHNMNVQNGKWDSDPSG TK App770 (1/87)
sp|P53601|A4_MACFA      MLPGLALLLLAAWTARA  LEVPTDGNAGLLAEPQIAMFCGRLNMHNMNVQNGKWDSDPSG TK MacQue (10/46)
sp|P79307|A4_PIG        MLPGLALVLLAAWTARA  LEVPTDGNAGLLAEPQVAMFCGKLNMHMNVQNGKWESDPSG TK Squirrel Monkey (1/8)
sp|Q60495|A4_CAVPO      MLPSLALLLLTTWTARA  LEVPTDGNAGLLAEPQIAMFCGKLNMHMNVQNGKWEPSDPSG TK Guinea Pig
sp|P08592|A4_RAT        MLPSLALLLLAAWTVRA  LEVPTDGNAGLLAEPQIAMFCGKLNMHMNVQNGKWESDPSG TK Rat (8/88)
tn|AAM90259             MLPSLALLLLAAWTVRA  LEVPTDGNAGLLAEPQIAMFCGKLNMHMNVQNGKWESDPSG TK Rat (8/88)
tn|AAR97726             MLPALALVLLASWTARA  LEVPTDGNAGLLAEPQVAMLCGKLRMHMNVQNGKWESDPLG TK Dog
sp|P12023|A4_MOUSE      MLPSLALLLLAAWTVRA  LEVPTDGNAGLLAEPQIAMFCGKLNMHMNVQNGKWESDPSG TK Mouse (14/89)
tn|AAH65529             MLPGLALLLLAAWTARA  LEVPTDGNAGLLAEPQIAMFCGRLNMHNMNVQNGKWDSDPSG TK App751
***.***:***:***.*** *****:***:***.*****:*** **

sp|P05067|A4_HUMAN      TCIDTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCL VG
sp|P53601|A4_MACFA      TCIDTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCL VG
sp|P79307|A4_PIG        TCIGTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHTHIVIPYRCL VG
sp|Q60495|A4_CAVPO      TCIGSKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCL VG
sp|P08592|A4_RAT        TCIGTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHTHIVIPYRCL VG
tn|AAM90259             TCIGTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHTHIVIPYRCL VG
tn|AAR97726             TCIGSKEDILQYCQEVY  PELQITNVVEANQPVTIQNWCKKGRKQCKTHAHIVIPYRCL VG
sp|P12023|A4_MOUSE      TCIGTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHTHIVIPYRCL VG
tn|AAH65529             TCIDTKEGILQYCQEVY  PELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCL VG
***.***:***:***.*** *****:***:***.*****:*** **

sp|P05067|A4_HUMAN      EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
sp|P53601|A4_MACFA      EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
sp|P79307|A4_PIG        EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
sp|Q60495|A4_CAVPO      EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
sp|P08592|A4_RAT        EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
tn|AAM90259             EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
tn|AAR97726             EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
sp|P12023|A4_MOUSE      EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
tn|AAH65529             EFVSDALLVPDKCKFLH  QERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDK FR
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sp|P05067|A4_HUMAN      GVEFVCCPLAEESDNVD  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVAEVE EE
sp|P53601|A4_MACFA      GVEFVCCPLAEESDNVD  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVAEVE EE
sp|P79307|A4_PIG        GVEFVCCPLAEESDNID  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVADVE EE
sp|Q60495|A4_CAVPO      GVEFVCCPLAEESDNID  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVADVE EE
sp|P08592|A4_RAT        GVEFVCCPLAEESDSID  SADAEEEDSDVWVGADTDYADGGEDKVVEVAEEEEVADVE EE
tn|AAM90259             GVEFVCCPLAEESDSID  SADAEEEDSDVWVGADTDYADGGEDKVVEVAEEEEVADVE EE
tn|AAR97726             GVEFVCCPLAEESDNID  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVADVE EE
sp|P12023|A4_MOUSE      GVEFVCCPLAEESDSVD  SADAEEEDSDVWVGADTDYADGGEDKVVEVAEEEEVADVE EE
tn|AAH65529             GVEFVCCPLAEESDNVD  SADAEEEDSDVWVGADTDYADGSEDKVVEVAEEEEVAEVE EE
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sp|P05067|A4_HUMAN      EADDDDEDDEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
sp|P53601|A4_MACFA      EADDDDEDDEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
sp|P79307|A4_PIG        EAEDDEDDEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
sp|Q60495|A4_CAVPO      EADDDDEDVEDGDEVEEE AEEPVEEATEKTTTSIATTTTTTTTESVEEVVREVCSEAETG PC
sp|P08592|A4_RAT        EAEDDEDVEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
tn|AAM90259             EAEDDEDVEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
tn|AAR97726             EAEDDEDDEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
sp|P12023|A4_MOUSE      EADDDDEDVEDGDEVEEE AEEPVEEATERTTSTATTTTTTTE SVEEVVREVCSEAETGPC
tn|AAH65529             EADDDDEDDEDGDEVEEE AEEPVEEATERTTSIATTTTTTTTESVEEVVREVCSEAETG PC
***.*** ***** *****:*** *****:*** *****:*** **

sp|P05067|A4_HUMAN      RAMISRWFVDVTEGKCA  PFFYGGCGGNRNNFDTEEYCMVCGSAMSQSLKKTQEPLA RD
sp|P53601|A4_MACFA      RAMISRWFVDVTEGKCA  PFFYGGCGGNRNNFDTEEYCMVCGSVMSQSLRKTREPLT RD

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sp|P79307|A4_PIG      RAMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCGSVMSQSLLKTTQEHL P QD
sp|Q60495|A4_CAVPO    RSMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCGSVMSQNLLKTSGE PVS QG
sp|P08592|A4_RAT      RAMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCGSVSSQSLLKTTSE PPLP QD
tn|AAM90259           RAMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCGSVSSQSLLKTTSE PPLP QD
tn|AAR97726           RAMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCGSVMSQSLLKTTQE PPLP QD
sp|P12023|A4_MOUSE    RAMISRWFYFDVTEGKCV PFFYGGCGGNRNNFDTEEYCMVCGSVSTQSLLKTTSE PPLP QD
tn|AAH65529           RAMISRWFYFDVTEGKCA PFFYGGCGGNRNNFDTEEYCMVCG -----

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sp|P05067|A4_HUMAN     PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
sp|P53601|A4_MACFA     PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
sp|P79307|A4_PIG       PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
sp|Q60495|A4_CAVPO     PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
sp|P08592|A4_RAT       PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
tn|AAM90259           PVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
tn|AAR97726           AVKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
sp|P12023|A4_MOUSE     PDKLPTTAASTPDAVDK YLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWE EAE R QA
tn|AAH65529           -SAIPTTAASTPDAVDKYLETPGDENEHAHFQKAKERLEAKH RERMSQVMREWE EAE RQA

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sp|P05067|A4_HUMAN     KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
sp|P53601|A4_MACFA     KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
sp|P79307|A4_PIG       KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
sp|Q60495|A4_CAVPO     KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
sp|P08592|A4_RAT       KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
tn|AAM90259           KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
tn|AAR97726           KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
sp|P12023|A4_MOUSE     KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL
tn|AAH65529           KNLPKADKKAVIQHFQE KVESLEQEAAANERQQLVETHMARVEAMLNDRRRLALENYIT AL

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sp|P05067|A4_HUMAN     QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
sp|P53601|A4_MACFA     QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
sp|P79307|A4_PIG       QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
sp|Q60495|A4_CAVPO     QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
sp|P08592|A4_RAT       QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
tn|AAM90259           QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
tn|AAR97726           QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
sp|P12023|A4_MOUSE     QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER
tn|AAH65529           QAVPPRPRHVFNMLKKY VRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIY ER

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sp|P05067|A4_HUMAN     MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
sp|P53601|A4_MACFA     MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
sp|P79307|A4_PIG       MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
sp|Q60495|A4_CAVPO     MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
sp|P08592|A4_RAT       MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
tn|AAM90259           MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
tn|AAR97726           MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
sp|P12023|A4_MOUSE     MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET
tn|AAH65529           MNQSLSLLYNVPAAVEE IQDEVDELLQKEQNYSDVLANMISEPRISYGNDALMP SLT ET

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sp|P05067|A4_HUMAN     KTTVELLPVNGEFSLDD LQPWHSFGADSV PANTENEVEPV DARPAADRGLTTRPGSGL TN
sp|P53601|A4_MACFA     KTTVELLPVNGEFSLDD LQPWHSFGADSV PANTENEVEPV DARPAADRGLTTRPGSGL TN
sp|P79307|A4_PIG       KTTVELLPVNGEFSLDD LQPWHFPFGVDSVPANTENEVEPV DARPAADRGLTTRPGSGL TN
sp|Q60495|A4_CAVPO     KTTVELLPVNGEFSLDD LQPWHFPFGVDSVPANTENEVEPV DARPAADRGLTTRPGSGL TN
sp|P08592|A4_RAT       KTTVELLPVNGEFSLDD LQPWHFPFGVDSVPANTENEVEPV DARPAADRGLTTRPGSGL TN
tn|AAM90259           KTTVELLPVNGEFSLDD LQPWHFPFGVDSVPANTENEVEPV DARPAADRGLTTRPGSGL TN
tn|AAR97726           KTTVELLPVNGEFSLDD LQPWHFPFGVDSVPANTENEVEPV DARPAADRGLTTRPGSGL TN

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sp|P12023|A4_MOUSE      KTTVELLPVNGEFLDD LQPWHPFGVDSVPANTENEVEPVDARPAADRGLTTRPGSGL TN
tn|AAH65529             KTTVELLPVNGEFLDD LQPWHSFGADSVANTENEVEPVDARPAADRGLTTRPGSGL TN
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sp|P05067|A4_HUMAN      IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
sp|P53601|A4_MACFA      IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
sp|P79307|A4_PIG        IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
sp|Q60495|A4_CAVPO      IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
sp|P08592|A4_RAT        IKTEEISEVKMDAEFRH DSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
tn|AAM90259             IKTEEISEVKMDAEFRH DSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
tn|AAR97726             IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
sp|P12023|A4_MOUSE      IKTEEISEVKMDAEFRH DSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
tn|AAH65529             IKTEEISEVKMDAEFRH DSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVI TL
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Bacterial cleavage site

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sp|P05067|A4_HUMAN      VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
sp|P53601|A4_MACFA      VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
sp|P79307|A4_PIG        VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
sp|Q60495|A4_CAVPO      VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
sp|P08592|A4_RAT        VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
tn|AAM90259             VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
tn|AAR97726             VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
sp|P12023|A4_MOUSE      VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
tn|AAH65529             VMLKKKQYTSIHGCVVE VDAAVTPEERHLSKMQQNGYENPTYKFFEOMQN
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